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Application Note #3

Principal Factors Influencing the Accuracy of FCS Data

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The major contributions to noise and uncertainty in Fluorescence Correlation Spectroscopy (FCS) data depend on correlation time. At all timescales, emission signal collection efficiency and fluorophore brightness are the primary factors influencing data quality. However, detector nonlinearities and correlator architecture are a factor in noise at lower correlation times. In this Application Note, I will discuss the most commonly observed contributions to noise in FCS data for various correlation time regimes and discuss strategies that can improve data accuracy.

A correlation function, $G(\tau)$, for a monodisperse sample in solution is shown in figure 1. For simplicity, I have divided the curve into three regions, depending on correlation time τ. In region I, detector afterpulsing causes a large background signal in the FCS data. In region II, Poisson noise, which is influenced by the correlation method, leads to a large variance. Finally, in region III, the signal to noise ratio is determined primarily by the particle brightness. Note that although typical time scales dividing the regions are shown in figure 1, these values will vary depending on correlator hardware and software, detector type, and fluorophore characteristics.

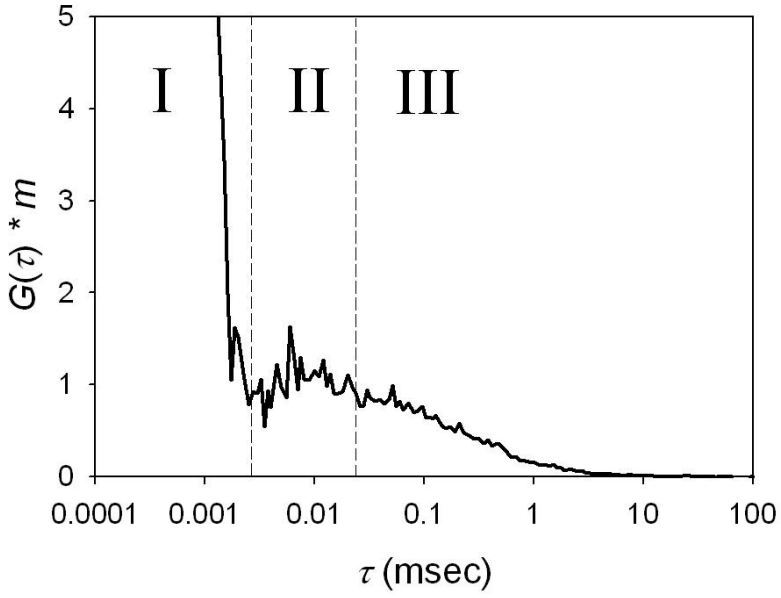


Figure 1: FCS data for a small, fluorescently-labeled protein in buffer. The x-axis has been divided into three regions (dotted lines) based on common sources of error in the correlation curve. *m* is the average number of particles in the focal volume.

Region I: Detector Afterpulsing

FCS experiments typically involve counting photons via avalanche photodiodes or photomultiplier tubes. However, these detectors often generate afterpulses, or spurious signals, shortly after the detection of a photon (Enderlein 2005). Because afterpulses are a response to an actual detection event, they are highly correlated to the initial occurrence and cause a large background in the measured FCS curve. For typical detectors used in our FCS experiments, afterpulses occur at lag times shorter than approximately 2.5 microseconds.

There are several methods for eliminating the afterpulsing signal, including filtering based on fluorophore lifetimes (Enderlein 2005), two-channel cross-correlation (Zhao 2003), and truncation of the correlation curve. In addition, new hybrid photon detectors that exhibit low or no afterpulsing are becoming available commercially, although current models have low quantum efficiencies (see, for example, model H8236-07 from Hammamatsu Corp., Bridgewater, New Jersey.)

Figure 2 shows FCS data obtained using a cross-correlation scheme to eliminate the afterpulsing signal. A beamsplitter was inserted into the emission pathway to divide the signal into two different photomultiplier tubes, and the fluorescence from the two channels was cross-correlated to remove afterpulsing signals. The correlation curve now contains information about short lag times that were previously masked by afterpulsing. However, the low τ signal is noisy. By eliminating afterpulsing contributions to $G(\tau)$, we have essentially extended Region II into lower correlation times. In addition, splitting the emission signal reduces the number of counts in each channel, increasing fluctuations and requiring increased time for data collection.

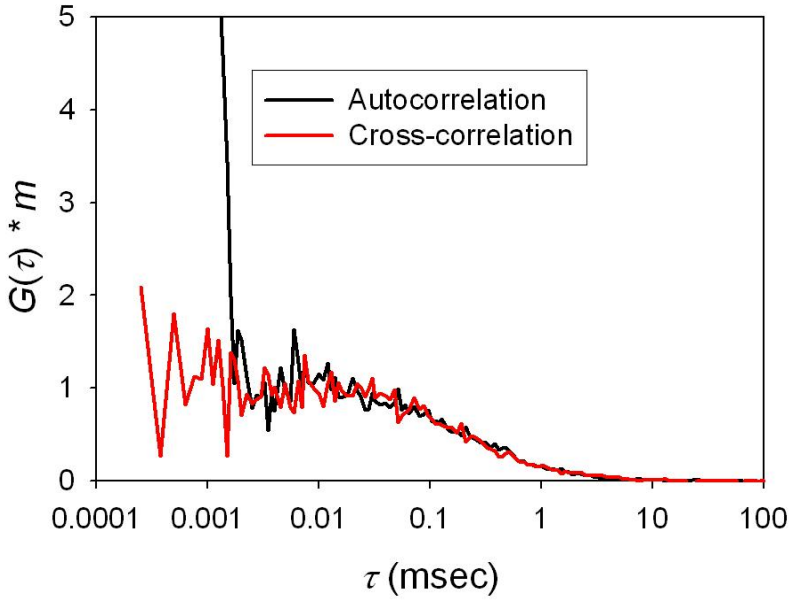


Figure 2: FCS data for fluorescently-labeled protein showing the removal of afterpulsing signals by splitting the emission between two detectors and cross-correlating. This data was obtained for the same sample shown in figure 1.

Region II: Poisson Noise

At the timescales represented by the correlation times in region II, particles are more or less stationary. Noise in this regime, called "Poisson noise" or "shot noise," is due to fluctuations in the random emission of photons from these fixed fluorophores. For FCS data, the signal to noise ratio depends on particle brightness and correlator bin size and is not affected by sample concentration. Poisson noise can be reduced by modifying correlation parameters, but at the cost of losing data at short correlation times.

Typical multi-tau correlator hardware divides photon stream data into time bins of a typical size Δt, which depends on the lag time (see Wohland 2001 for a detailed description). Therefore, the correlation curve is generated via the function $g(\nu)$, which is defined by (Saffarian 2003):

$$g(\nu) = \frac{\frac{1}{(N-\nu)}\sum_{i=1}^{N-\nu} n_i n_{i+\nu}}{\left(\frac{1}{(N-\nu)}\sum_{i=\nu}^{N} n_i\right)\left(\frac{1}{(N-\nu)}\sum_{i=1}^{N-\nu} n_i\right)} \qquad (1)$$

Here, N is the total number of time bins of size Δt measured during the experiment, while n_i is the number of photons detected in the i^{th} bin. ν is proportional to the lag time, i.e. $\tau = \nu \Delta t$. The reported correlation function, $G(\tau)$, is given by $G(\tau) = \langle g(\tau / \Delta t) \rangle - 1$.

The variance of $g(\nu)$ can be found by plugging $n_i = \langle n \rangle + \delta n_i$ into equation (1) and calculating $\langle g(\tau) \rangle$ and $\langle g^2(\tau) \rangle$. A detailed derivation can be found in Saffarian 2003. When particle movement is low and the illumination intensity is low enough that laser-induced photochemistry is not a factor, the mean time between photons is much greater than the fluorophore lifetime, and we can assume that the probability of photon emission is not time-dependent. Therefore, for stationary fluorophores, $\langle \delta n_i^2 \rangle = \langle n \rangle$, where $\langle n \rangle$ is the average number of counts in a bin. In addition, this Poisson noise is uncorrelated, so $\langle \delta n_i \, \delta n_j \rangle = 0$ if $i \neq j$. For large N, the variance simplifies to:

$$\mathrm{var}(g(\nu)) = \frac{1}{N\langle n \rangle^2} \qquad (2)$$

Assuming the experiment duration is T, we have $N \approx T/\Delta t$. The average number of counts in a bin of size Δt is $\langle n \rangle = m \cdot \sigma \Delta t$, where σ is the mean fluorescence for a single fluorophore and m is the number of fluorophores in the focal volume. Plugging these values into equation 3, we have:

$$\mathrm{var}(g(\nu)) = \frac{1}{T\Delta t \cdot m^2 \sigma^2} \quad (3)$$

In region II, the FCS signal is approximately $G(0) = 1/m$. Therefore, the signal to noise ratio in this regime is:

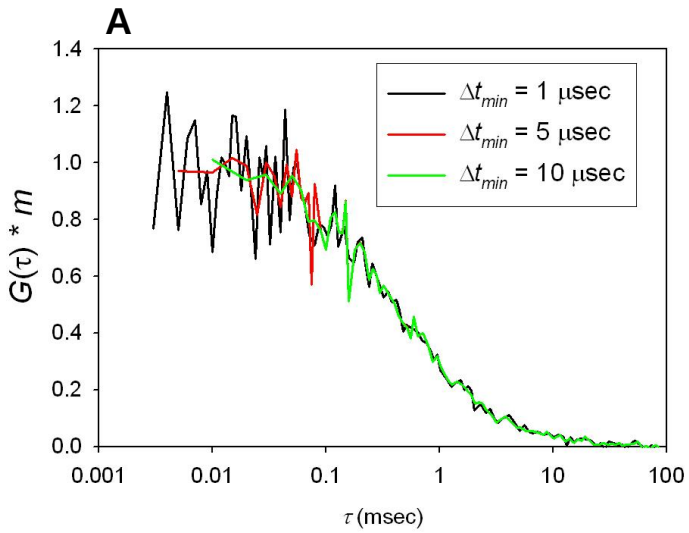
$$\left(\frac{S}{N}\right)_{II} = \frac{1/m}{\sqrt{\mathrm{var}(g(\nu))}} = \sigma\sqrt{T\Delta t} \quad (4)$$

This ratio does not involve the number of particles in the focal volume, and therefore is not dependent on concentration. The signal is also weakly dependent on the total experiment time T and the bin size Δt.

Reducing the noise in region II involves choosing a bright fluorophore and optimizing the detection pathway to maximize σ. In addition, the bin size Δt can be adjusted using software correlation, but increasing Δt also increases the minimum lag time τ that can be measured.

For multi-tau correlation schemes, which are used in most commercially available hardware correlators, Δt is a function of τ. The first sixteen data points are generated using a bin size Δt_{min}, while the next eight data points use a bin size of $2\Delta t_{min}$, the following eight $4\Delta t_{min}$, and so forth. This increase in bin size with τ provides a quasi-logarithmic range of lag times that cover a large span of timescales (Wohland 2001). Therefore, the smoothness of the curve for a multi-tau correlation strategy will depend on the minimum bin size, and shot noise will be decreased for increasing τ.

Figure 3A shows how Δt_{min} affects the correlation curve for a monodisperse fluorophore in solution. All curves were generated using the same raw photon stream data and correlation was accomplished using the software program BurstAnalyzer via a multi-tau algorithm. This software is available for free at http://www.drbio.cornell.edu/software_burst_dev.html. Note that the correlation curve in 3A is smoother for increasing minimum bin sizes, but that some low-τ data is lost for higher Δt_{min}. The effect of increasing Δt_{min} on the goodness of the fit is shown in figure 3C. The R^2 value and the uncertainties in parameter estimates are improved as Δt_{min} increases. This analysis suggests that it is desirable to use the largest Δt_{min} that is compatible with the range of timescales of interest in a given FCS experiment.



B

$$G(\tau) = \frac{1}{m}\left(1+\frac{\tau}{\tau_C}\right)^{-1}\left(1+\frac{\tau}{\alpha^2\tau_C}\right)^{-1/2}$$

C

Δt_{min} (μsec)	R^2	τ_c (msec)	m
1	0.95	0.412 +/- 0.047	144.0 +/- 2.7
5	0.98	0.402 +/- 0.025	143.2 +/- 2.0
10	0.99	0.404 +/- 0.019	143.7 +/- 1.8

Figure 3: The effect of increasing Δt_{min} on FCS data for rhodamine green in solution. **A:** The same raw photon trace was analyzed using various Δt_{min}, resulting in smoother curves as Δt_{min} increases. **B.** The equation showing the theoretical correlation curve for a monodisperse sample. τ_C is the correlation time and α is related to the shape of the focal volume (see Saffarian 2003.) **C.** Result of fitting the data in A to the equation in B using nonlinear regression via SigmaPlot.

Hardware correlators most often use a small, fixed minimum bin size. For example, the ALV-7002 hardware correlator (ALV-Laser, Vertriebsgesellschaft m.b.H., Langen, Germany) uses Δt_{min} = 100 nanoseconds, while the correlator.com Flex02-02D model correlator has Δt_{min} = 2.5 nanoseconds (Correlator.com, Bridgewater, New Jersey). These small bin sizes enable measurement of features that occur at small correlation times, but in many FCS experiments, the shortest timescale of interest is in the microsecond, rather than nanosecond, range, making this high resolution unnecessary. In addition, these small bin sizes increase Poisson noise contributions to the signal.

Region III: Correlated Fluctuations Noise

Fluorophores diffuse through the focal spot at the timescales that characterize region III. Because diffusion is a random process, particle dwell times will be subject to Poisson statistics. Therefore, the stochastic nature of the dwell times ensures variance in the measurement for finite experiment durations. In addition, fluctuations due to random fluorescence emission compete with particle number fluctuations, leading to additional noise in the signal (Koppel 1974.)

For a large number of average particles in the focal volume, the signal to noise ratio is approximately (Qian 1990):

$$\left(\frac{S}{N}\right)_{III} \approx \frac{q\sqrt{N}}{\sqrt{1+q^2}} \tag{5}$$

where N is the number of time bins collected and q is the average number of photons collected from a single fluorophore in the time Δt. Using $N \approx T/\Delta t$, and $q = \sigma\Delta t$, we have:

$$\left(\frac{S}{N}\right)_{III} \approx \frac{\sigma\sqrt{T\Delta t}}{\sqrt{1+\sigma^2\Delta t^2}} \tag{6}$$

As fluorophore brightness increases, this approaches $\sqrt{N} \approx \sqrt{T/\Delta t}$, the stochastic limit for the fluctuation measurement. For a dim particle, the signal to noise ratio approaches the value seen in region II (equation 4).

In region III, the optimal bin size Δt is slightly larger than the mean lag time between photon arrivals, on the order of tens to hundreds of microseconds for a 10-100 kHz signal. This requirement is satisfied for most hardware correlators.

Equations 5 and 6 are approximate (see Saffarian 2003 for a more complete expression.) However, they capture essential features of noise in region III. Note that this analysis is incorrect for very large and small numbers of particles in the focal volume. When m is large, the FCS signal, which is proportional to $1/m$, becomes too small to measure. In addition at very low concentrations, the signal to noise ratio is proportional to $\sqrt{m}$ (Koppel 1974). Hence, FCS measurements are most reliable at concentrations in the 1 nanomolar – 1 micromolar range; in this regime, the signal to noise ratio is essentially independent of m.

Conclusion

The signal to noise ratio for FCS data is primarily determined by the brightness of a single fluorophore, the total measurement time, and the bin size used to generate the correllellogram. Under normal experimental conditions, sample concentration is not a factor.

Particle brightness, determined by fluorophore cross section and emission pathway efficiency, is the single most important factor in FCS data quality. Brightness increases with laser intensity, but laser-induced photophysics, including triplet excitation and photobleaching, become problematic at high intensities. Consequently, fluorophore photostability is a primary consideration in FCS experiments. In addition, reducing loss in the detection pathway by using high-quality filters and a high-NA objective lens will reduce noise.

Increasing the experiment duration has a modest effect on noise because the variance in the correlation curve is proportional to $1/\sqrt{T}$ for most τ. Reducing the variance by half requires a fourfold increase in experiment duration, which is difficult or unrealistic for many experiments.

Low-τ Poisson noise (region II) is the most problematic source of variance in a typical FCS experiment. Under normal conditions, the region III curve is smooth while region II data is very noisy. Therefore, larger correlation bin sizes are desirable for many experiments. Software or hardware correlation systems that allow tuning of Δt may help minimize this source of error.

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